Comp. Biochem. Physiol. Vol. 113B, No. 1, pp. 73–77, 1996

ISSN 0305-0491/96/$15.00
SSDI 0305-0491(96)02001-2

Stress Proteins Elicited by Cold Shock in the Biting Midge, *Culicoides variipennis sonorensis* Wirth and Jones

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ABSTRACT. *In vivo* protein expression in the abdominal viscera of C. *v. sonorensis* was examined from adult flies that were cold shocked for various lengths of time at 0, −10, or −15°C and labelled at 25°C with ^{35}S-methionine at 0, 2, 4 and 6 hr during the recovery period. *In vitro* labelling showed that seven unique proteins (23, 40, 43, 48, 60, 70 and 92 kDa) were produced in C. *v. sonorensis* exposed to low temperatures *in vivo*. In general, the rate of expression and quality of stress proteins were directly proportional to both the severity and duration of the cold shock. A polyclonal antibody to the moth hsp 60/63 crossreacted with antigen from the viscera of the 60 kDa protein that was expressed during recovery from cold shock. This crossreaction with C. *v. sonorensis* suggests that the 60 kDa protein expressed during recovery from cold shock in the midge is immunologically related to the moth heat shock protein (hsp). Weather records from central Wyoming suggest that if the stress proteins produced by C. *v. sonorensis* enhance survival of the earliest, normally lethal temperatures (e.g., −5°C), populations of these insects can persist for an additional 20–30 d in an average year and thus extend the time they can transmit bluetongue virus. COMP BIOCHEM PHYSIOL 113B,1:73–77, 1996.

KEY WORDS. Stress proteins, cold shock, heat shock, *Cullicoides variipennis*

INTRODUCTION

Organisms that are exposed to elevated temperatures synthesize a small number of highly conserved proteins collectively known as heat shock proteins (Lindquist, 1986; Schlesinger, 1986; Petersen and Mitchell, 1985), although perhaps more accurately termed stress proteins. These proteins are produced in response to many types of environmental and metabolic stresses (Tatem and Stollar, 1989; Barettino *et al.*, 1988; Hiromi *et al.*, 1986), and they appear to be involved in mechanisms that allow organisms to survive physiological challenge (Joplin *et al.*, 1990). Some insects exhibit similarities in their physiological responses to high and low temperatures. For example, cold shock injury and mortality in *Drosophila melanogaster*, *Sarcophaga crassipalpis* and *Culicoides variipennis sonorensis* can be greatly reduced by brief exposure to a moderately low temperature or a relatively high temperature (Nunamaker, 1993; Burton *et al.*, 1988; Chen *et al.*, 1987).

Culicoides v. sonorensis is the primary vector of the bluetongue (BLU) viruses in the United States (Tabachnick, 1992). The physiological and biochemical responses of this insect to extreme temperatures may be important in understanding the epizootiology of BLU viruses. Evidence suggests that post-parous C. *v. sonorensis* can be found in the adult stage, at low densities, well before the established phenology of the insect should have allowed overwintering larvae to emerge, pupate and feed (F. Holbrook, personal communication). This observation is consistent with the possibility that adult C. *v. sonorensis* may have the capacity to overwinter. Overwintering adult vectors harboring BLU virus would significantly accelerate the seasonal epizootiology of the pathogen in temperate areas. Because the virus has not been isolated from overwintering larvae, infected adults could not otherwise be present until mid-March (Barnard and Jones, 1980). Adult C. *v. sonorensis* are protected from severe cold by a moderate cold shock (Nunamaker, 1993). Adult capacity to survive subfreezing temperatures by virtue of cold shock significantly expands the temporal and geographic limits of disease transmission. Given a 2 week generation time for C. *variipennis* (Barnard and Jones, 1980), moderate extensions of survival via cold hardening are epidemiologically significant. The purposes of the present study were to examine relationships between C. *v. sonorensis* heat shock and cold shock responses to determine if: (1) low temperatures will elicit the expression of stress proteins and (2) unique proteins are produced in response to low temperature exposure.

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Received 18 November 1994; accepted 22 May 1995.

MATERIALS AND METHODS

Insects

Culicoides v. sonorensis specimens were from a colony established in 1957 (Jones, 1960). The insects were reared at 20°C, 40–60% RH, and a photoperiod of 13:11 (L:D) hr. Adults were maintained under the same conditions for 48 hr and were allowed to feed on an aqueous solution of 10% sucrose.

Temperature Treatment

Adult female flies, 48 ± 4 hr old, were placed in 1.5 ml microfuge tubes which were then placed into a 0, −10 or −15°C circulating bath that consisted of a 1:1 mixture of polyethylene glycol and water. At specified intervals of 2 hr (for −10 and −15°C), 8 hr (for −10 and 0°C) or 16 hr (for 0°C), flies were removed from the cold treatment and held at 25°C for recovery. Although all specimens appeared to be alive at the end of the recovery period, some may have been mortally injured and would not have survived more than several hrs. Control flies were maintained continuously at 25°C. Heat treatments were performed in the same manner but using a thermostatically-controlled water bath set at 43°C.

Protein Labelling of Insect Tissue

Following the low temperature treatments, flies were maintained at 25°C for specified recovery times of 0, 2, 4 and 6 hr. Each *in vitro* culture was prepared by removing the viscera from the abdomens of 40 flies (in insect ringer solution), and placing these preparations into microfuge tubes containing 30 μl of Grace's medium without methionine (Grace, 1962). The samples of viscera were not homogenized due to the small surface to volume ratio. Preliminary studies indicated that homogenization of these small pieces of tissue resulted in cell disruption, thereby hindering the uptake of radioisotope. Each microfuge tube received 1 μl (10 mC/ml) Tran^{35}S-label (culture medium) and was incubated without agitation for 2 hr at 25°C. Preparations from control flies were labelled for 1, 2, 4, or 6 hr. Three replications of each treatment were performed.

Protein Electrophoresis

The culture medium was removed and 30 μl of sample buffer (0.06% Tris, 10% glycerol, 2% SDS, 10% B-mercaptoethanol, 0.01% PMSF, bromophenol blue) was added. The tubes were placed in a boiling water bath for 4 min, centrifuged in a microfuge for 1 min at 14,000 rpm and held at −80°C until needed. The amount of label that was incorporated into protein was determined by the trichloroacetic acid (TCA) precipitation method (Joplin and Denlinger, 1990).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) was performed using a 0.75 mm thick 12% polyacrylamide resolving gel and a 4% stacking gel. Equal TCA precipitable counts were loaded in each well. Gels were run at 15mA/gel for 4.5 hr, stained for 2 hr in 40% EtOH, 7% acetic acid and 0.125% Coomassie Brilliant Blue R-250, and destained overnight in 10% EtOH, 7% acetic acid and 10% glycerol. Gels were than placed in Enhance® (DuPont NEN Research Products, 549 Albany St., Boston, MA 02118) for 1 hr, rinsed in water for 30 min and dried in a vacuum gel drier for 2 hr at 60°C. The dried gels were put on Kodak X-Omat AR film for 2 weeks at −70°C.

Western Blots

All Western blotting was performed according to the BioRad Immuno-Blot® Assay Procedure (Bio-Rad Laboratories, 2000 Alfred Nobel Drive, Hercules, CA 94547). Antigen was applied to the nitrocellulose membrane by electrophoretic transfer. The primary antibody (Product No. SPA-805 from StressGen Biotechnologies Corp. 120-4243 Glanford Ave., Victoria, BC, CANADA V8Z 4B9), a rabbit anti-hsp 60/63 polyclonal made against moth testicular antigen, has been shown to exhibit a wide range of cross-reactivity in most vertebrates, invertebrates, and procaryotes (Miller, 1987).

RESULTS

Lethal Temperature

The lethal temperature for 48-hr old flies from the laboratory colony was determined to be −17°C; no fly survived a 2-hr exposure at this temperature (Fig. 1).

Effects of Cold Shock on Protein Expression

In vivo protein expression in the abdominal viscera was examined from adult flies that were cold shocked for various lengths of time at 0, −10 or −15°C and labelled at room temperature (RT) with ^{35}S-methionine at various times during the recovery period.

Proteins expressed in flies treated at −15°C for 2 hr and labelled for 1 hr immediately after cold shock, and at intervals up to 6 hr later, are shown in Fig. 2. The heat shock proteins

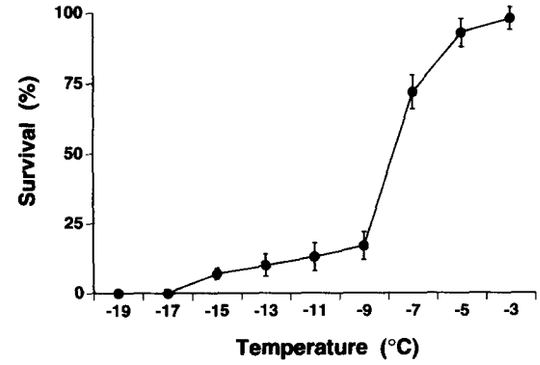

FIG. 1. Survival of adult *Culicoides v. sonorensis* transferred directly to 20°C to a given temperature for 2 hr. Data expressed as the mean (± SE) survivorship of five replicates of 50 flies each.

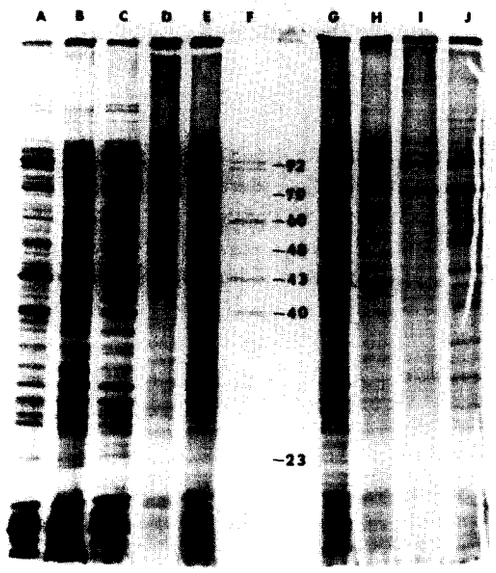

FIG. 2. Proteins expressed in viscera from *Culicoides v. sonorensis* abdomens during recovery from cold shock. Adult flies were cold shocked prior to labelling viscera for 2 hr as described in Materials and Methods. Flies exposed to −15°C for 2 hr and viscera labelled 2 hr at 25°C (A) immediately after cold shock, (B) 2 hr after cold shock, (C) 4 hr after cold shock, or (D) 6 hr after cold shock; (E) 25°C control; (F) 43°C for 2 hr, then labelled at 25°C for 1 hr. Flies exposed to −10°C for 2 hr and viscera labelled 2 hr at 25°C (G) immediately after cold shock, (H) 2 hr after cold shock, (I) 4 hr after cold shock, of (J) 6 hr after cold shock. Numbers are the molecular weights in kDA of the proteins discussed in the text.

of 40, 43, 60 and 92 kDa that were observed in the 43°C heat shock control also were expressed immediately after the insects were returned to 25°C from the −15°C exposure. The 40, 43 and 60 kDa proteins continued to be expressed through 4 hr of recovery at 25°C, but by 6 hr following cold shock they were no longer detected by gel electrophoresis (Fig. 2). The 92 kDA stress protein continued to be expressed even after 6 hr at 25°C. Three proteins with molecular weights of 23, 48 and 70 kDA were present in the abdominal viscera after cold shock, but were not found in the 25°C viscera controls or in any of the heat shock controls. Like the cold shock induced stress proteins, these proteins were expressed immediately after cold shock. The 23 and 48 kDa proteins were not expressed after 6 hr of recovery at 25°C, whereas the 70 kDa protein was most intense after 6 hr (Fig. 2).

Proteins expressed in flies treated at −10°C for 2 hr and labelled for 1 hr following cold shock differed markedly from those elicited at −15°C, with a general diminution in the intensity of expression (Fig. 2). The 92 kDa stress protein was strongly expressed immediately after cold shock but less so after 2, 4 or 6 hr at 25°C. The 40, 43 and 60 kDa proteins were only slightly expressed under these conditions. The 23 and 48 kDa proteins were apparent immediately after cold shock, but were not detected after 2 hr at 25°C. The 92 kDa protein was observed throughout the recovery period. The 70 kDa protein was expressed immediately after cold shock, diminished after 2 hr at 25°C, and then was a predominant protein after 6 hr of recovery (Fig. 2).

In flies treated at −10°C for 8 hr, the 70 and 92 kDa proteins were heavily expressed throughout the recovery period (0 to 6 hr at 25°C; Fig. 3). Flies treated at 0°C for 8 or 16 hr and held at 25°C for up to 6 hr produced the 92 kDa protein throughout the recovery period. The 40, 43, 48 and 92 kDa proteins were consistently expressed during all recovery times, with some tendency to declining production with increasing recovery time. However, the 23, 60 and 70 kDa proteins were most evident after 6 hr of recovery in flies that were subjected to 0°C for 8 hr (Fig. 3).

Among the major proteins that were synthesized 0 to 4 hr following cold shock, three (23, 48 and 70 kDa) were not observed in either the 25°C controls or in flies that had been heat shocked (43°C for 2 hr). The 70 kDa protein was most abundant during the final hours of the recovery period.

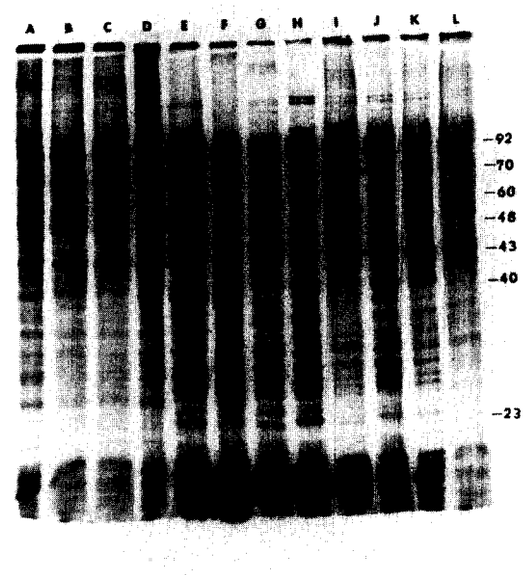

FIG. 3. Proteins expressed in viscera from *Culicoides v. sonorensis* abdomens during recovery from cold shock. Flies exposed to −10°C for 8 hr and viscera labelled 2 hr at 25°C (A) immediately after cold shock, (B) 2 hr after cold shock, (C) 4 hr after cold shock, or (D) 6 hr after cold shock; Flies exposed to 0°C for 8 hr and viscera labelled 2 hr at 25°C (E) immediately after cold shock, (F) 2 hr after cold shock, (G) 4 hr after cold shock, or (H) 6 hr after cold shock. Flies exposed to 0°C for 16 hr and viscera labelled 2 hr at 25°C (I) immediately after cold shock, (J) 2 hr after cold shock, (K) 4 hr after cold shock, or (L) 6 hr after cold shock. Numbers are the molecular weights in kDA of the proteins discussed in the text.

Identification of Heat Shock Protein 60 by Western Blotting

The 60 kDa protein expressed during recovery from heat shock or cold shock in C. *v. sonorensis* is immunologically related to a moth heat shock protein that was isolated and purified by *Stress*Gen Biotechnologies Corporation. In Western blotting experiments, we demonstrated that a polyclonal antibody to the moth heat shock 60/63 protein crossreacted with antigen from viscera obtained from C. *v. sonorensis* abdomens (Fig. 4).

DISCUSSION

Given that mortality in C. *v. sonorensis* is induced by temperatures as high as −5°C, it is apparent that cold hardening could markedly extend the survival and phenology of adult vector populations. Even the warmest sites in Wyoming have a daily low temperature of −18°C (the condition necessary to cause 100% mortality in C. *v. sonorensis*) an average of >1 d per month from November through March. At moderate elevations in central Wyoming (e.g., Casper, 1,580 m asl) temperatures of −7°C (which induces 25% mortality in adult flies) may occur (1% of the time) in early September, and lows of -10°C are not uncommon (10% of the time) in mid October (Martner, 1986). Variation in microhabitat temperatures simply adds spatial heterogeneity to the temporal pattern reflected in weather station data. Thus, across microhabitats we would expect that early freezes are commonly followed by extended periods of warmer weather, during which continued feeding, mating and egg production may occur. Habitats that can support C. *v. sonorensis* generally have 20–30 d between the earliest lethal low temperatures and the average date by which these temperatures occur (Martner, 1986).

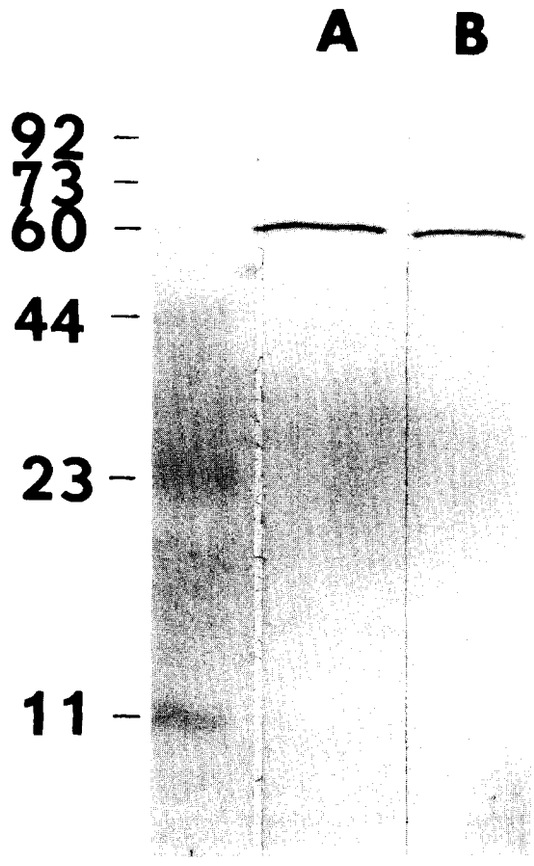

FIG. 4. Western blot of protein from the abdominal viscera from *Culiocoides v. sonorensis* that were induced with cold shock using an antibody to a moth testicular heat shock protein (hsp 60). (A) Flies exposed to −15°C for 2 hr and viscera labelled for 2 hr at 25°C, 2 hr after cold shock and (B) Flies exposed to 43°C for 2 hr and viscera labelled for 1 hr at 25°C, 2 hr after heat shock. The position of hsp 60 and the molecular weight standard markers are identified.

In vitro labelling showed that stress proteins were produced in the abdominal viscera of *Culicoides* that were exposed to low temperatures *in vivo*. Although C. *v. sonorensis* is protected from cold shock injury by a moderate heat or cold shock (Nunamaker, 1993), we have no direct evidence that the stress proteins are responsible for protection against cold-induced injury. In their study of *Drosophila melanogaster*, Burton *et al.* (1988) demonstrated stress protein expression during recovery from a 14 hr exposure to 0°C. Joplin *et al.* (1990) reported the induction of hsps (stress proteins) in *Sarcophaga crassipalpis* after extended exposure to 0°C, but they noted that expression was more pronounced during recovery from a brief exposure to more severe temperatures (−10 to −18°C). Proteins specific to cold shock have been reported in *S. crassipalpis*, and include 23, 45 and 78 kDa proteins (Joplin *et al.*, 1990).

Heat shock is nearly always accompanied by a drastic reduction in the synthesis of proteins that are expressed at normal temperatures (Petersen and Mitchell, 1985). During recovery from cold shock, however, normal protein synthesis proceeds along with enhanced production of stress proteins. This has been demonstrated in the present study, and earlier by Burton *et al.* (1988) and Joplin *et al.* (1990). These findings all suggest that stress protein synthesis and the repression of normal protein synthesis are not interdependent.

We found that the level of stress protein expression in *Culicoides* that were exposed to a lower temperature for a short time (e.g., −15°C for 2 hr) was nearly identical to that expressed in *Culicoides* exposed to a less severe temperature for a longer time (e.g., 0°C for 8 hr). We noted that stress proteins were rapidly expressed when the tissue was returned to 25°C, and that the amount of stress protein expression, though not linear, was temperature-dependent. Exposure to −15°C for 2 hr or 0°C for 8 hr resulted in large amounts of stress protein expression, whereas very little stress protein was expressed following 2 or 8 hr of exposure to −10°C, and the timing of expression differed markedly from 0 to −15°C.

We suggest that the 70 kDa protein is a generic response to cold; it is readily elicited by any drop in temperature. Furthermore, exposing the flies to −15°C for 2 hr approaches the lethal conditions that were established at the onset. The anomalous protein production under these conditions could

reflect the early stages of death or perhaps severe disruption of physiological and biochemical processes associated with lethal temperatures. We do not know whether these anomalous data would persist if the insects were first exposed to 5°C (i.e., cold-hardened) and then subjected to −15°C. It is likely that −10°C for 2 hr may have been too short and/or mild of an exposure to elicit the full complement of stress (cold shock) proteins, even though these conditions were adequate to elicit cold hardening (Nunamaker, 1993). It is apparent that time of exposure interacts with degree of stress, and some minimum levels of each of these factors must be reached if full biochemical responses to cold are to be realized.

Although we have demonstrated that low temperatures elicit the expression of stress proteins and several unique cold shock proteins, it is not possible to conclude from these data whether any of these proteins offer thermal protection to the insect. Future studies will examine the relationships between stress proteins and the survival of C. *v. sonorensis* that have been exposed to low temperature. This research will utilize genetic lines of C. *v. sonorensis* that exhibit varying degrees of tolerance to cold temperature.

We thank J. G. Wigington and J. A. Johansson for expert technical assistance, J. A. Kempert for providing specimens of Culicoides variipennis sonorensis, *and D. Raver for help with preparation of the manuscript. D. L. Dahlman, F. R. Holbrook, G. J. Hunt, R. E. Lee, W. J. Tabachnick and W. C. Wilson provided helpful discussions and critical comments on the manuscript.*

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